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FOR:

SEPARATION PARTICLES

INVENTOR:

MICHAEL ALAN REEVE

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SEPARATION PARTICLES

Field of the Invention

The present invention relates to magnetic particles for the separation and manipulation of biological compounds. More particularly, the invention relates to streptavidin-derivatised colloidal Fe_3O_4 particles.

Background of the Invention

Many molecular biological methods make use of the capture and solid-phase manipulation of compounds. Such methods include solid-phase DNA sequencing, DNA/RNA hybridisation, separation of polymerase chain reaction (PCR) products, labelling of single-stranded nucleic acid probes, gene assembly, *in vitro* mutagenesis, yeast artificial chromosome (YAC)-screening, DNA cloning, sequence-specific purification of DNA/RNA binding proteins, cell separation and isolation of bacteria etc.

A number of capture methods rely on the interaction between biotin and biotin-binding proteins such as avidin or streptavidin. Streptavidin is a preferred biotin-binding protein as it has four identical subunits each of which has a high affinity binding site for biotin making it suitable for use in the rapid and efficient isolation of biotin-labelled target molecules. The appropriate biotinylated compounds vary according to the application but include compounds such as double-stranded and single-stranded DNA, RNA, proteins, sugars and lectins.

A popular method for such capture and solid-phase manipulation relies upon the use of streptavidin-coated monodisperse magnetic particles, such as those supplied by Dynal A.S. (Dynabeads^{TM} M-280 Streptavidin) which are highly uniform, superparamagnetic, polystyrene beads coated with a polyurethane layer. The stability of the interaction between biotin and streptavidin enables manipulations such as DNA strand melting, hybridisation and elution to be performed without affecting the immobilisation of the biotinylated compound while the magnetic particles allow separation through exposing the beads to a magnetic field gradient. However, such beads are expensive and cumbersome to produce.

Other methods are known in the art. Thus WO 96/37313 discloses complex magnetically responsive microparticles which can be coated with surface-active molecules, such as streptavidin, to effect separation of biological materials. Similarly, US 5,693,539 describes polymer-coated magnetic particles which may be conjugated to binding moieties such as avidin or biotin for the purification and/or separation of bio-molecules. Biotinylated lipid-coated magnetic nanoclusters bearing avidin surface residues have also been described in the literature for use in affinity capture (Sonti S.& Bose A., Colloids and Surfaces (1997) 8 (4); pp 1999-204). However, as with the DYNABEADS™ M-280 Streptavidin product described above, the preparation of these magnetic particles or nanoclusters is both complex and expensive.

There is therefore a need to provide a simple composition of magnetic particles for the separation and manipulation of biological compounds that can be prepared more readily and more cheaply than the known products.

Summary of the Invention

In view of the needs of the art, the present invention provides a composition comprising colloidal Fe_3O_4 particles coated with a biotin-binding protein.

The present invention further provides a method of immobilising a biotinylated compound comprising incubating said biotinylated compound in solution in the presence of a composition of the present invention.

Brief Description of the Drawing

Figure 1 shows an image of single-stranded and double-stranded PCR products run out on an agarose gel stained with Vistra Green.

Detailed Description of the Preferred Embodiments

Accordingly, in a first aspect of the present invention, there is provided a composition comprising colloidal Fe_3O_4 particles coated with a biotin-binding protein. Suitably, the biotin-binding protein is avidin or streptavidin. In a particularly desirable embodiment, the biotin-binding protein is streptavidin.

It has been found that colloidal Fe_3O_4 particles can be synthesised and that such particles can be coated with a biotin-binding protein such as streptavidin. Such coated particles have a high iron content (approximately 72%) aiding the speed and efficiency of magnetic separations. They also display excellent performance for the capture and solid-phase manipulation of biotinylated compounds such as biotinylated PCR product strands. Such particles are particularly suitable for automated processes.

Desirably, the Fe_3O_4 particles in accordance with the first aspect of the invention are suspended in aqueous solution. Suitably, Fe_3O_4 particles may be suspended in water or in an aqueous solution comprising phosphate at a concentration of less than 2mM or comprising NaOH at a concentration of less than 1mM. Other suitable aqueous solutions may comprise HCl at a concentration of less than 1mM, BSA at less than 100μg/ml, NaCl at less than 5M, dNTP at less than 1mM, urea at less than 8M or SDS at less than 1%.

The sizes of the particles will be highly dispersed and dependent on the solvent conditions. Particles are at their smallest effective size i.e. most disaggregated after repeated washing in deionised water.

In a second aspect of the invention, there is provided a method for synthesis of a composition in accordance with the first aspect, the method comprising incubating colloidal Fe_3O_4 particles with a biotin-binding protein, preferably streptavidin or avidin and, most preferably, streptavidin.

In one embodiment of the second aspect, the method comprises the steps of:

a) forming colloidal Fe_3O_4 particles by mixing aqueous $FeCl_2$ with aqueous $FeCl_3$ and adding aliquots of the mixture to an alkaline solution;

b) adding a biotin-binding protein.

In a particularly desirable embodiment, the biotin-binding protein added in step b) is streptavidin.

Suitably aqueous $FeCl_2$ is mixed with aqueous $FeCl_3$ in suitable proportions for the formation of Fe_3O_4. In one embodiment, aqueous $FeCl_2$ is mixed with aqueous $FeCl_3$ at a molar ratio of between 1:1.5 and 1:2, $FeCl_2$: $FeCl_3$, and preferably at a ratio of 1:1.5. In a particularly preferred embodiment, aqueous $FeCl_2$ may be in the form of $FeCl_2.4H_20$ and aqueous $FeCl_3$ may be in the form of $FeCl_3.6H_2O$. Suitably, the alkaline solution to which the mixture of $FeCl_2$ and $FeCl_3$ is added is an ammonia solution.

The biotin-binding protein is desirably added in excess i.e. to provide an excess of biotin-binding protein compared to the number of available sites that can bind a biotin-binding protein. The number of available binding sites will be dependent on the available surface area of Fe_3O_4 particles.

In a third aspect of the invention, there is provided a method of immobilising a biotinylated compound comprising incubating said biotinylated compound in a solution in the presence of a composition in accordance with the first aspect.

Suitable conditions for the binding of proteins such as avidin or streptavidin to biotin will be recognised by those skilled in the art. Preferably, binding of the protein to biotin occurs when the Fe_3O_4 particles coated with a biotin-binding protein are incubated with the biotinylated compound at room temperature.

In one embodiment of the third aspect, the biotinylated compound is selected from a nucleic acid molecule, a protein or a peptide. Suitable nucleic acid molecules include double stranded or single stranded DNA, PCR products, oligonucleotides, RNA or PNA. Suitably, a protein or peptide may be an antibody or antibody fragment.

In another embodiment, the biotinylated compound may be a linker compound such as a biotinylated linker arm containing a reactive group at the opposite end to the biotin. Suitable linker compounds include sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce) and (+) biotinyl-3-maleimidopropionamidyl-3,6-dioxaoctainediamine. The reactive group may subsequently be reacted to allow linkage to another compound, for example, to glutathione or a nickel chelating agent which can, in turn, be bound to a GST- or His-tagged compound.

In a yet another embodiment, the biotinylated compound and the composition are subsequently magnetically attracted to a surface and separated from said solution.

The following examples are for illustration purposes only and should not be used in any way to limit the appended claims.

EXAMPLE 1

a) Synthesis of colloidal Fe_3O_4 particles

4 g of $FeCl_2.4H_20$ (Sigma, F-2130, lot #99F3495) were added to 30 ml of water (Elga, Spectrum, >18 MΩ). 20 ml of 1 M HCl (AR/964 26897 pp62) were then added. 8.85 g of $FeCl_3.6H_20$ (Aldrich, 20,792-6, lot #45469) were finally added to the above with stirring at room temperature until dissolved.

50x 1 ml aliquots of the acidified mixed iron chlorides were slowly added to 500 ml of water (Elga, Spectrum, >18 MΩ) containing 25 ml of concentrated ammonia solution (BDH, AnalaR, 10012, lot #7198610M) with constant stirring to give an 8 mg/ml stock of Fe_3O_4 in dilute ammonia solution.

250 ml of the 8 mg/ml stock of Fe_3O_4 in dilute ammonia solution was allowed to settle under gravity and the supernatant was carefully decanted until the volume was 100 ml (giving a 20 mg/ml Fe_3O_4 stock in dilute ammonia solution).

2x 50 ml aliquots of the 20 mg/ml Fe_3O_4 in dilute ammonia solution were placed in 50 ml Falcon tubes. The Fe_3O_4 particles were washed twice with 50 ml of water (Elga, Spectrum, >18 MΩ) using a pair of magnetic Dynal MPC-1 separators (Dynal, product #12001) to give a 20 mg/ml aqueous stock of colloidal Fe_3O_4 particles.

b) Streptavidin coating of colloidal Fe_3O_4 particles

5 mg of streptavidin (Sigma, S-4762, lot #27H6824) was taken up in 1.92 ml of water to give a 2.6 mg/ml stock (stored at 4°C). 67 µl of 2.6 mg/ml streptavidin were added to 10 ml of the 20 mg/ml aqueous stock of colloidal Fe_3O_4 particles followed by rolling overnight at 4°C.

After overnight incubation, samples were washed twice in 10 ml of water. Particles were then resuspended in 10 ml of water. Particles were stored at 4°C.

EXAMPLE 2

Probing the linkage between the Fe_3O_4 particles and streptavidin to various chemical treatments

In order to probe the sensitivity of the linkage between the Fe_3O_4 particles and streptavidin to various chemical treatments, the streptavidin-coated colloidal Fe_3O_4 particles were pre-treated in 20 µl at 20 mg/ml for 1 hour at 4°C in the solutions shown in Table 1:

Table 1. Solutions Used for Samples

Sample	Solution
1	Water
2	Water
3	200 mM phosphate buffer (pH 7.4)
4	20 mM phosphate buffer (pH 7.4)
5	2 mM phosphate buffer (pH 7.4)
6	200 μM phosphate buffer (pH 7.4)
7	5 M NaCl
8	500 mM NaCl
9	50 mM NaCl
10	5 mM NaCl
11	1 M NaOH
12	100 mM NaOH
13	10 mM NaOH
14	1 mM NaOH
15	1 M HCl
16	100 mM HCl
17	10 mM HCl
18	1 mM HCl
19	1 mM dNTPs
20	100 μM dNTPs
21	10 mg/ml BSA
22	1 mg/ml BSA
23	100 μg/ml BSA
24	10 μg/ml BSA
25	8 M urea
26	6 M urea
27	4 M urea

28	2 M urea
29	10 % SDS
30	1 % SDS
31	0.1 % SDS
32	0.01 % SDS

After pre-treatment, the particles were washed twice in 500 µl of water and were then taken up in 100 µl of 20 mM tris-HCl (pH 7.4), 2 mM EDTA (pH 8.0), 2 M NaCl (i.e. at 4 mg/ml) ready for biotin capture of PCR product strands.

PCR amplification

PCR reactions were carried out as described below:

(-40) Forward Strand Primer =

5' GTTTTCCCAGTCACGACG 3' (SEQ ID No. 1)

(-28) Reverse Strand Primer-with 5' biotin (batch number 3-5022-4/4) =

5' biotin-AGGAAACAGCTATGACCAT 3' (SEQ ID No. 2)

Both primers were obtained from MWG Biotech GmbH with biotin labelling of the reverse strand primer by incorporation during synthesis.

target AM =

5'GTTTTCCCAGTCACGACGACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGC CGTTACTGCCATGGTCATAGCTGTTTCCT 3' (SEQ ID No. 3)

reaction volume	100 µl
(-40) FSP	5 pmol per reaction
(-28) RSP-with 5' biotin	5 pmol per reaction
AM target	5 fmol per reaction

dNTP concentration	50 μM final
25 mM $MgCl_2$	2 μl per reaction
units of *Taq* polymerase	2 U per reaction

25 cycles of

97°C for 1 minute
50°C for 2 minutes
72°C for 3 minutes

followed by

72°C for 5 minutes

96x PCR reactions were carried out. After PCR amplification, samples were pooled and 100 μl aliquots were used for biotin capture assay.

Capture and solid-phase manipulation of biotinylated PCR product strands

100 μl of the pooled PCR reaction was mixed with an equal volume of 4 mg/ml streptavidin-coated colloidal Fe_3O_4 particles taken up in 20 mM tris-HCl (pH 7.4), 2 mM EDTA (pH 8.0), 2 M NaCl. The tube was incubated at room temperature for 30 minutes with mixing.

The streptavidin-coated colloidal Fe_3O_4 particles were then washed with 500 μl of 10 mM tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 1 M NaCl. Three more identical washes were performed.

The washed streptavidin-coated colloidal Fe_3O_4 particles were finally incubated in 100 µl of 0.1 M NaOH for 10 minutes at room temperature to denature double-stranded PCR products. The supernatant was removed and added to 100 µl of 0.5 M HEPES.

Upon completion of the programme, 20 µl aliquots from 200 µl of PCR product single strands were mixed with 10 µl of 50 % glycerol AGE loading dye and electrophoresed on a 1.5 % agarose gel in 1x TBE.

The gel was stained for 60 min in 500 ml of 1x TBE containing 50 µl of Vistra Green (Amersham, RPN 5786, lot #3163-7).

The stained gel was imaged on a Fluorimager SI (LSRDNT1) with a 488 nm laser, 570 DF 30 filter and a PMT setting of 700 V.

Results

The PCR product single strands prepared from the PCR reactions were run out on a gel next to double-stranded controls in order to assess the yield of single-stranded DNA prepared. Note that double-stranded DNA PCR products (2 µl of 1x PCR reaction) are loaded at 1/5th the amount of the single-stranded DNA PCR products (20 µl of 0.5x PCR reaction). The results of this analysis are shown in the gel image in Figure 1 in which the top row has dsDNA PCR product (A), samples 1-20, dsDNA PCR product (A) and the bottom row has dsDNA PCR product (A), samples 21-32, dsDNA PCR product (A).

As there is a large difference between the staining efficiency between double-stranded DNA (which stains well), and single-stranded DNA (which stains poorly) with the intercalating agent Vistra Green, some double-stranded DNA carry-over is visible in the gel image. Thus, the double-stranded DNA carry-over appears more marked. Some of this carryover can, however, be accounted for as the surface of the Fe_3O_4 particles has a high affinity for compounds with phosphate groups and thus will bind PCR products. The

binding is reversed by subsequent alkali treatment, whereupon single stranded material of both (+) and (-) sense is released, reanneals, and stains with Vistra Green.

The sensitivity of the linkage between uncoated Fe_3O_4 particles and streptavidin to various chemical treatments has been investigated. The linkage is found to be sensitive to: phosphate concentrations greater than 2 mM, NaOH concentrations greater than 1 mM, HCl concentrations greater than 1 mM and BSA concentrations greater than 100 μg/ml. The linkage is, however, found to be resistant to: NaCl concentrations up to 5 M, dNTP concentrations up to 1 mM, urea concentrations up to 8 M and SDS concentrations up to 1%.

While the preferred embodiment of the present invention has been shown and described, it will be obvious in the art that changes and modifications may be made without departing from the teachings herein. The matter set forth in the foregoing description and accompanying figure is offered by way of illustration only and not as a limitation. Although a number of embodiments are described in detail in the above examples, the instant invention is not limited to such specific examples. The actual scope of the invention is intended to be defined in the following claims when viewed in their proper perspective based on the prior art.